BIO-BLEACHING OF PULP USING LACCASE, MEDIATOR, AND CHAIN TRANSFER AGENT

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/318,292, filed on September 10, 2001.

FIELD OF INVENTION

The present invention relates to the use of chain transfer agents that, in combination with laccase and a mediator, can be used to bleach pulp.

BACKGROUND OF THE INVENTION

Paper pulp is typically processed from wood through the Kraft (and other) processes. The process produces a pulp with a dark brown color, mostly due to the presence of lignin and lignin derivatives. For many applications, the lignin has to be removed by a process known in the art as "bleaching." This is typically done commercially in several stages in pulp mills, wherein lignin is first oxidized and then removed from the pulp.

Recently, several research groups have been working with enzymes to biologically bleach pulp, referred to as "bio-bleaching." Bio-bleaching is a methodology whereby an enzyme is used to decrease the optical brightness and/or lignin content of the pulp or paper. The standard measure of bleaching efficiency is "Kappa number." Enzymes that have most commonly been used include laccase, lignin peroxidase, and manganese peroxidase. An enzyme group that has received particular attention is the laccase family of enzymes, which are copper-containing enzymes that are known to be good oxidizing agents in the presence of oxygen. Laccases are found in microbes, fungi, and higher organisms.

For many applications, the oxidizing efficiency of a laccase can be improved through the use of a mediator that enhances the activity of the laccase. Systems that include a laccase and a mediator are known in the art as laccase-mediator systems (LMS). There are several known

mediators for use in laccase-mediator systems. These include HBT (1-hydroxybenzotriazole), ABTS [2,2'-azinobis(3-ethylbenzothiazoline-6-sulfinic acid)], NHA (N-hydroxyacetinilide), NHAA (N-acetyl-N-phenylhydroxylamine), HBTO (3-hydroxy-1,2,3-benzotriazin-4(3H)-one), and VIO (violuric acid). In addition, there are several compounds containing NH-OH or N-O that have been found to be useful as mediators. There remains a need, however, to increase the bleaching efficiency of bio-bleaching systems.

SUMMARY OF THE INVENTION

The invention provides the use of chain transfer agents in standard laccase-mediator systems (LMS). These agents themselves are not necessarily mediators that enhance the activity of the enzyme. However, the chain transfer agents have been found to enhance the bleaching efficiency of certain laccase/mediator systems.

The invention provides a process of bleaching a lignin-containing material. The process comprises the step of treating the material with an oxidative enzyme, a mediator that enhances the oxidative activity of the enzyme, and a chain transfer agent. In another embodiment, the invention provides a process of oxidizing a substrate comprising treating the substrate with an oxidizing enzyme, a mediator that enhances the oxidative activity of the enzyme, and a chain transfer agent. The invention also provides a composition comprising an oxidative enzyme, a mediator that enhances the oxidative activity of the enzyme, and a chain transfer agent.

In one embodiment of the invention, the enzyme has laccase activity. In another embodiment, the enzyme is a laccase, a catechol oxidase, a monophenol monooxygenase, a bilirubin oxidase, or a mixture thereof. In yet another embodiment, the invention further comprises adding a hydrolase, such as xylanase. The process of the invention can further include the addition of an oxidizing agent, such as air, oxygen, and/or hydrogen peroxide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention discloses the use of chain transfer agents to enhance the bleach efficiency of laccase/mediator systems. Laccase itself can bleach pulp only to a limited extent. Certain mediators can enhance the activity of laccase in pulp bleaching. It has been discovered that some mediators are effective in bleaching an organic dye but are not effective in bleaching pulp. In fact, in some cases the Kappa numbers are found to increase after treating the pulp with laccase and one of these mediators.

One likely rationalization of this surprising finding entails the nature of laccase. Whereas the LMS can oxidize and degrade lignin and thereby achieve bleaching, it can also polymerize lignin under suitable reaction conditions. The oxidation, degradation, and polymerization reactions compete with one another. In this scenario, it is then possible to have an LMS that gives a high activity for the dye assay (predominant reaction being oxidation), but an increase in Kappa number (if polymerization is the major reaction).

The above observation reminded us of a concept in free radical chemistry and polymer science. Vinyl polymerization can be considered to consist of four reactions: (i) initiation; (ii) propagation; (iii) chain transfer; and (iv) termination. In all four reactions, free radicals are involved. One common method to cut down the molecular weight of a polymer during polymer synthesis is to use chain transfer agents. These agents do not destroy the free radicals, but instead terminate a growing polymer chain, and start a new chain.

In view of the above reasoning, we believe the use of a chain transfer agent may solve this problem. Chain transfer agents have not been used previously in combination with mediators. In fact, the role of chain transfer in bleaching has not been previously appreciated. It is noteworthy that a chain transfer agent does not generate free radicals by itself, nor does it trap free radicals. Chain agents are described, for example, by G. Scott in Chapter 4, Antioxidants: Radical Chain-Breaking Mechanism from "Atmospheric oxidation and antioxidants", Elsevier Publishing Co., New York, NY, p. 115.

In one embodiment, the mediator of the invention is a hardwood black liquor or a softwood black liquor. In another embodiment of the invention, the chain transfer agent is anthracene, carbon tetrabromide, or a mixture thereof.

The process disclosed herein is not confined to bio-bleaching of pulp. Other laccase-catalyzed (or laccase-facilitated) oxidation reactions or polymerization may also be enhanced with the addition of chain transfer agents. Examples of chain transfer agents can be found in "Polymer Handbook", Second Edition, Editors J. Brandrup and E. H. Immergut, J. Wiley & Son, New York, NY, 1975, pages II-57 thru II-104.

The mediators and chain transfer agents of this invention are capable of enhancing the activities of laccase and laccase-related enzymes for the purpose of pulp bleaching. In the context of this invention, these enzymes include laccase enzymes of the enzyme classification EC 1.10.3.2, catechol oxidase enzymes of the enzyme classification EC 1.10.3.1, the monophenol monooxygenase enzymes of the enzyme classification EC 1.14.99.1, and bilirubin oxidase enzymes of the enzyme classification EC 1.3.3.5. The EC (Enzyme Commission) number is based upon the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB).

The laccase in this invention may be derived from microbial, fungal, or other sources. It may furthermore be produced by recombinant methods, such as cultivating a host cell transformed with a recombinant DNA vector which includes a DNA sequence encoding the laccase (and DNA sequences encoding functions that permit the expression of laccase DNA sequence) in a culture medium under the conditions that permits the expression of the laccase, and recovering the enzyme from the culture.

Another aspect of the invention provides a process for oxidizing a substrate that comprises treating the substrate with a composition comprising an enzyme exhibiting laccase

activity and an enzyme enhancing agent. The enzyme enhancing agent can be selected from one or the above described enzyme enhancing agents.

The enhancing agent may be present in concentrations of from about 0.01 micromolar to about 1000 micromolar, more preferably from about 0.1 micromolar to about 250 micromolar and most preferably from about 0.5 to about 100 micromolar.

The chain transfer agent may be present in concentrations of from about 0.01 micromolar to about 1000 micromolar, more preferably from about 0.1 micromolar to about 250 micromolar and most preferably from about 0.5 to about 100 micromolar. The enzyme is used in amounts of from about 0.1 to 400 units (defined in Examples using ABTS as substrate) for 1 g dry pulp, more preferably from 1 to 200 units and even more preferably from about 10 to 100 units and most preferably from 20 to 50 units.

The process of the invention can further include the step of adding an oxidizing agent, such as at least one of air, oxygen, and hydrogen peroxide.

One embodiment of the invention provides a process for bleaching a lignin-containing material that comprises treating the material with an enzyme exhibiting laccase activity and an enzyme enhancing agent. In this embodiment of the invention, the enhancing agent may be present in an amount of from about 0.1% to about 15% based on the weight of the dry lignin containing material, more preferably from about 0.1% to about 10% and even more preferably from about 0.5% to about 5% and most preferably from about 1% to about 4 %. One example of a lignin containing material is wood pulp. The process for bleaching a lignin-containing material can further include the step of adding an oxidizing agent, such as at least one of air, oxygen, and hydrogen peroxide.

EXAMPLES

The following examples are illustrative of the present invention, and are not intended to be construed in any way as limiting the scope of the invention.

Laccase Enzyme Assay

In the examples, two *Aspergillus* laccases have been used, both from Novozymes A/S (Denmark). NovoSample 51002 works best at pH 4-5 while NovoSample 51003 works best at pH 5-6. Enzyme dosage has been found to have an effect on bio-bleaching. For example, 0.1 ml of the 51003 laccase gives a modest Kappa number reduction when HBT is used, and a huge reduction when ABTS is used.

The specific activity was determined using ABTS (0.5 mM) as substrate. One unit of activity is equal to the umol of the oxidized product from ABTS per min per mg protein at pH 6.0 at 23 °C. The extinction coefficient of the oxidized ABTS is: ε(max) at 420 nm=36,000M-1cm-1.)

Alternatively, the activity of laccase (NS51003) was determined using syringaldazine as substrate. In this case, one unit of activity is equal to the change of 0.001 UV absorbance at A530nm per minute per ug protein in 2 ml of 100 mM, pH 5.5 potassium phosphate buffer, and 0.5 ml of 0.25 mM syringaldazine in methanol at 23 °C.

Kappa Number

Delignification of the pulp was measured as the change in Kappa number according to theTAPPI method T236 cm-85. A typical procedure is as follows. A known mass of paper pulp (containing lignin) is reacted with an excess of potassium permanganate in an acid solution for a specified period of time to oxidize the lignin. After the reaction, the residual permanganate is determined by titration. The Kappa number is defined as the volume (ml) of 0.1N potassium

permanganate consumed by 1 g of moisture-free pulp in 0.5N sulfuric acid after a ten-minute reaction time at 25 °C under conditions such that one-half of the permanganate remains unreacted. A linear relationship with the lignin content of the pulp and the measurement of the Kappa number has already been done on samples as low as 300 mg of pulp.

Pulp Bleaching

A softwood Kraft pulp, Kappa number 31.0, was treated with a laccase (NS51003) under the following conditions:

Enzyme dosage	45 units /g pulp
pH	5.5
Temperature	50 °C
Reaction time	16 hours
Pulp consistency	2%

The dried pulp was added to 80 ml of 50 mM phosphate, pH 5.5, and disintegrated in a blender. The pulp was then transferred to a 500 ml conical flask and the blender was washed with 20 ml of the same buffer. The washed buffer and the pulp were combined. The mediator was added at 1-4% (w/w, based on the dry pulp), followed by the addition of the laccase. The pH of the pulp mixture was adjusted to 5.5 if needed. The flask was covered with an aluminum foil with holes punched through and incubated at 50 °C for 16 hrs on a rotary shaker at 200 rpm.

After the enzymatic treatment, the pulp mixture was filtered through a Buchner funnel, and the pulp was washed with water. The pulp from the control experiment was treated at the same pH and temperature as described above.

The washed pulp was then treated with an alkaline solution under the following conditions:

Pulp	2 g
Water	200 ml
NaOH	240mg
H_2O_2 (30%)	400ul
Temperature	70°C
Reaction time	3 hours

The filtered pulp was repulped in the alkaline solution and incubated at 70 °C for 3 hrs. The pH of the pulp mixture should be between 11.7-12.00 during the entire treatment. After the treatment, the pulp mixture was filtered through a Buchner funnel, and the pulp was washed with water extensively and then dried in a hood overnight.

The delignification of the pulp was measured as a change in Kappa Number according to TAPPI method T236 cm-85.

Example 1 - Chicago Blue Assay Using Hardwood Black Liquor And Softwood Black Liquor As Laccase Mediators

In the following example, black liquor from hardwood (oak) and softwood (pine) were tested with laccase to quantify the degree of enhancement that the black liquor impart to laccase. The Chicago Blue Dye, also known as Direct Blue 1 or DB1, was used for this assay. The Chicago Blue Dye is fully described by Schneider et al. in U.S. Patent No. 5,885,304, which is hereby incorporated by reference.

Each of the compounds (i.e., potential mediators) was dissolved in water, or in ethanol if the potential mediator was not water soluble, and then mixed with a phosphate buffer and a Chicago Blue solution. A solution of a laccase was added to make up 1 ml of the final solution,

containing 20 uM mediator, 20 mM buffer at pH 5.5 or 7.0, 0.1-1% laccase (v/v) and Chicago Blue solution, with absorbance at A610 nm between 0.6 to 0.8. The change in the absorbance at A610 nm was measured immediately using a UV-VIS spectrophotometer (UV-1201, Shimadzu Scientific Instruments) after the enzyme was added. The decrease in absorbance was recorded at 30-second intervals for 5 minutes and was used to estimate the efficiency of the mediator.

An oak black liquor and a pine black liquor (from Hercules, Inc., Durango, Georgia) were used as mediators to enhance laccase-catalyzed bleaching of Chicago Blue in solution. Both stock solutions contain 14-15% solids. Additional samples were made via acid treatment.

The acid-treated black liquors were prepared using the following procedure: (1) adjust pH of 20 ml of the black liquors to 1.0 using conc. HCl; (2) incubate the acidified solution at 80C for 1 hr; (3) cool down to room temperature; (4) adjust the pH to neutral (6.5-7.5) using 5 M NaOH solution.

The following are the results from the bleaching of the Chicago Blue dye. It is clear that the black liquor samples are good mediators for laccase as far as discoloration reaction is concerned.

Mediator (0.1umole/ml)	Usage (ugram)	ΔmA610 (3 min) at pH 5.5	ΔmA610 (3 min) pH 7.0
none		0	0
ABTS	10	445	341
Oak black liquor	100 (14%)	228	370
Pine black liquor	100 (14%)	26	18
Acid-treated oak black liquor	100 (13%)	108	225

Example 2 - Pulp Bleaching

In this example, we used the typical pulp bleaching experiment as described above. We also used laccase as the enzyme, hardwood black liquor (HWBL) as the mediator, and two chain transfer agents (anthracene and CBr_4).

Laccase	Mediator	Chain Transfer Agent	Pulp	Kappa No.
0	0	0	2 g	31
100 ul	0	0	2 g	28.0
100 ul	HBT, 20 mg	0	2 g	26.1
100 ul	HWBL, 200 ul	0	2 g	28.0
100 ul	HWBL, 200 ul	anthracene, 20 mg	2 g	25.4
100 ul	HWBL, 200 ul	CBr_4, 20 mg	2 g	24.4

The data above clearly indicate that the black liquor alone is not effective in enhancing pulp bleaching (although the black liquor is a mediator for the discoloration reaction of Chicago Blue dye). However, in the presence of a chain transfer agent, a noticeable decrease in Kappa numbers is obtained. Thus, the combination of black liquor and a chain transfer agent is indeed an effective mediator mixture for the enhancement of pulp bleaching.

It is to be understood that the above described embodiments are illustrative only and that modification throughout may occur to one skilled in the art. Accordingly, this invention is not to be regarded as limited to the embodiments disclosed herein.